Virology **294,** 209–221 (2002)
doi:10.1006/viro.2001.1295, available online at http://www.idealibrary.com on IDEAL®

Simian Immunodeficiency Viruses with Defective *nef* Genes Show Increased Susceptibility to the Noncytotoxic Antiviral Activity of $CD8^+$ Lymphocytes

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Received June 1, 2001; returned to author for revision August 7, 2001; accepted November 9, 2001

The noncytotoxic soluble factor produced by $CD8^+$ T cells inhibits replication of HIV and SIV *in vitro* and is thought to play a crucial role in combatting infection *in vivo.* We determined the effect of human $CD8^+$ lymphocytes on the *in vitro* replication potential of both wild-type and *nef*-defective mutants of the simian immunodeficiency virus SIVmac251. Although replication of wild-type SIVmac251 in unstimulated human PBMC supplemented with IL-2 was unaffected by the presence of $CD8^+$ T cells, the *nef* mutants were susceptible to the inhibitory effects. The effect of exogenous IL-2 depended upon the culture conditions: (i) in nonstimulated human PBMC depleted of $CD8^+$ T cells, addition of IL-2 had a positive effect on the growth of the *nef*-defective viruses; (ii) in total human PBMC, IL-2 appeared to reinforce the $CD8^+$ T-cell-dependent inhibition of the same mutant viruses. This strongly suggests that IL-2 stimulates the noncytotoxic anti-HIV/SIV response of $CD8^+$ cells present in PBMC cultures. PHA stimulation of unfractionated human PBMC overrode the suppression of viral replication by $CD8^+$ T cells. Depletion of activated T cells expressing the IL-2 receptor α-chain ($CD25^+$ T cells), present in small amounts in these primary T cell cultures, dramatically reduced viral replication, indicating that the depleted cell population harbors the target cells permissive for viral replication. Furthermore, using neutralizing antibodies we could show that inhibition by the β-chemokines MIP-1α, MIP-1β, and RANTES and the inhibitory effect of $CD8^+$ lymphocytes on *nef* mutant SIVmac viruses are harbored on different levels.

Key Words: control of virus replication; HIV/SIV suppressing activity; *in vitro* replication system.

INTRODUCTION

Human (HIV) and simian (SIV) immunodeficiency viruses carry genes encoding Nef, a conserved cytoplasmic protein of 25 to 36 kD, which associates with the plasma membrane through an N-terminal myristate (Franchini *et al.,* 1986; Kaminchik *et al.,* 1991; Myers *et al.,* 1993). Nef is expressed as an early protein from abundant, multiply spliced viral mRNA (Klotman *et al.,* 1991). Several groups have shown that Nef has a positive effect on viral replication in peripheral blood mononuclear cells (PBMC) (Terwilliger *et al.,* 1991; DeRonde *et al.,* 1992; Zazopoulos and Haseltine, 1993) and macrophages (Murphy *et al.,* 1993; Miller *et al.,* 1994). This enhancement of replication appears to reflect, at least partly, an increase in the intrinsic infectivity of virus particles (Chowers *et al.,* 1994; Aiken and Trono, 1995; Miller *et al.,* 1995; Schwartz *et al.,* 1995) through stimulation of proviral DNA synthesis (Aiken and Trono, 1995; Schwartz *et al.,* 1995). Furthermore, Nef alters several cellular functions. It downregulates the surface expression of CD4 (Garcia and Miller, 1991; Aiken *et al.,* 1994; Rhee and Marsh, 1994; Sanfridson *et al.,* 1994) and major histocompatibility complex class I molecules (Schwartz *et al.,* 1996). Nef also seems to modulate T cell activation events in the host cell (Luria *et al.,* 1991; Niederman *et al.,* 1992; Skowronski *et al.,* 1993; Baur *et al.,* 1994; Spina *et al.,* 1994; Du *et al.,* 1995).

The importance of Nef is clearly shown by the fact that functional SIV *nef* expression is required to maintain high viral loads in infected rhesus macaques (Kestler *et al.,* 1991) and by the rapid reversion of point mutations in the *nef* gene *in vivo* (Whatmore *et al.,* 1995). Although macaques immunized with SIV carrying extensive deletions in the *nef* gene (SIVΔ*nef*) are usually fully protected from infection with the pathogenic wild-type (wt) virus (Kestler *et al.,* 1991), infection with these forms of live attenuated vaccine virus can lead to high levels of viremia and disease progression in both infant and adult macaques (Baba *et al.,* 1995, 1999; Connor *et al.,* 1998). In addition, although a cohort of humans infected with a naturally occurring *nef*-deletion variant of HIV-1 maintained low virus loads and remained healthy for at least 14 years (Deacon *et al.,* 1995), it has recently been reported that some of these patients are meanwhile showing signs of $CD4^+$ cell loss (Learmont *et al.,* 1999).

The mechanisms controlling virus replication in the host are unknown. Depletion of $CD8^+$ T cells in rhesus infected with SIVΔ*nef* is associated with a 1–2 log increase in plasma viremia (Metzner *et al.,* 2000), suggest-

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0042-6822/02 $35.00

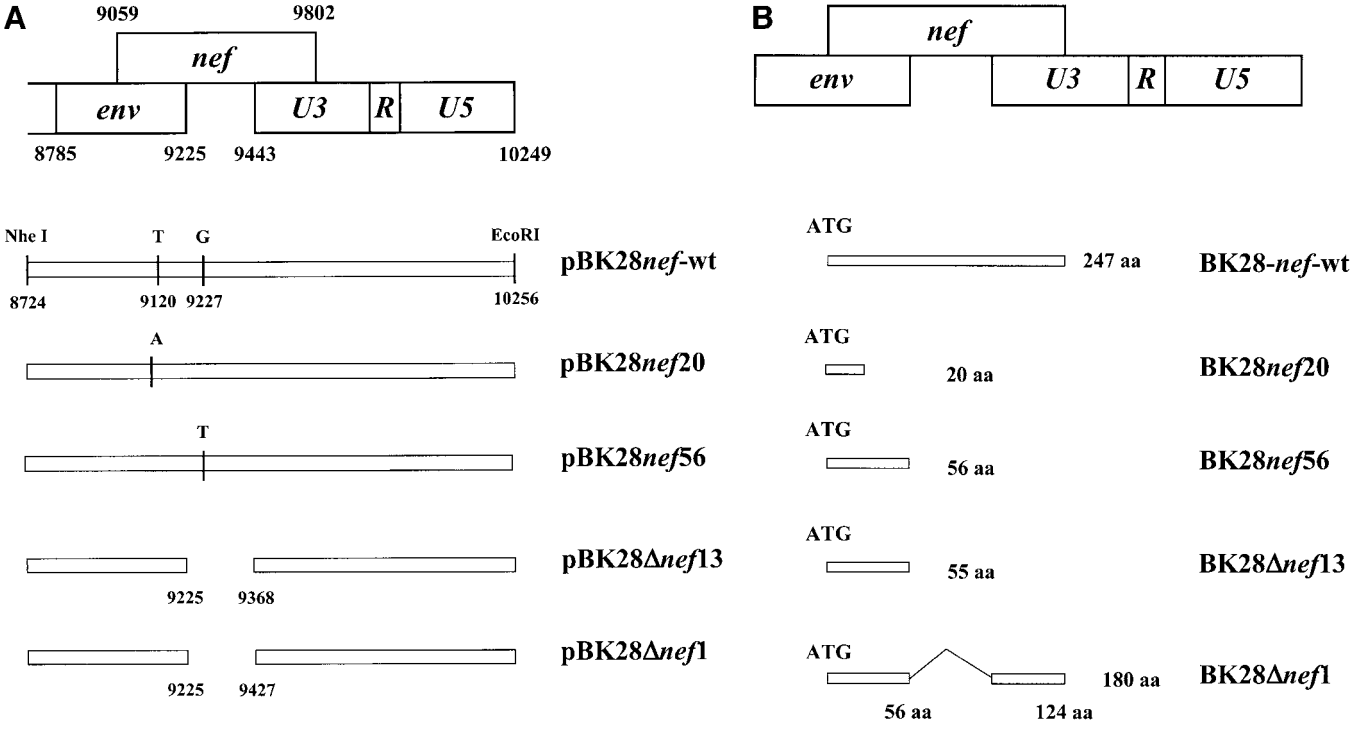

FIG. 1. (A) SIVmac251-BK28 *nef* alleles. A premature stop codon is located in the *env* gene at position 8785. (B) SIVmac251-BK28 mutant Nef proteins.

ing that these cells play an important role in controlling viral replication. Two types of $CD8^+$ T-cell-mediated antiviral functions have been described in HIV infection: one involves the classical antigen-specific HLA-restricted cytolysis of infected cells (Walker and Plata, 1990), and the second inhibits HIV replication in the absence of cell killing (Wiviott *et al.,* 1990; Walker *et al.,* 1991; Levy *et al.,* 1996). This noncytolytic antiviral response was first identified *in vitro* with lymphocytes from HIV-seropositive patients (Walker *et al.,* 1986) and correlates with the clinical state of the infected individual (Mackewicz *et al.,* 1991). The noncytotoxic antiviral activity is mediated primarily by activated $CD28^+$ $CD8^+$ cells (Landay *et al.,* 1993; Barker *et al.,* 1997), which are not restricted by major histocompatibility complex (MHC) class I. They are active against a wide variety of HIV strains, can suppress HIV replication in both $CD4^+$ cells and macrophages (Barker *et al.,* 1998), and are associated with the production of soluble $CD8^+$ cell antiviral factor (Levy *et al.,* 1996). $CD8^+$ T cells secrete potent antiviral factors able to suppress HIV/SIV replication *in vitro* (Chen *et al.,* 1993; Levy, 1993; Ennen *et al.,* 1994; Baier *et al.,* 1995; Cocchi *et al.,* 1995; Mackewicz *et al.,* 1995; Blackbourn *et al.,* 1997), which may play an important role in controlling the virus *in vivo* (Cocchi *et al.,* 1996). However, neutralization of these factors by antibodies does not totally abrogate the antiviral effect of $CD8^+$ cell supernatant, which suggests that the complete repertoire of antiviral factors still remains unknown (Clerici *et al.,* 1996; Moriuchi *et al.,* 1996).

Considering the negative influence of $CD8^+$ cell-mediated antiviral activity and the positive influence of Nef, we examined the interaction of those two. The replication behavior of the SIVmac251 molecular clone BK28, carrying a complete *nef* open reading frame, and a series of corresponding mutants defective in *nef* was investigated under various conditions in unfractionated and $CD8^+$ T-cell-depleted human PBMC. Since blood from rhesus macaques was not available in sufficient amounts and sequence analyses have revealed that SIVmac shows a 99% homology to HIV-2 (Chakrabarti *et al.,* 1987), suggesting a common origin (Gao *et al.,* 1992; Mansfield *et al.,* 1995), blood from healthy human donors was used. As the Nef protein influences the activation state of the host cell (Skowronski *et al.,* 1993; Du *et al.,* 1995), the experiments were, in addition, performed using primary human T cells in different states of activation.

RESULTS

SIVmac251-BK28 *nef* alleles

Starting with the molecular clone BK28 of SIVmac251, *nef*-defective mutants were generated by PCR assembly (Figs. 1A and 1B; Table 1). BK28*nef*20 has a single nucleotide exchange at position 9120 (TTG to TAG) resulting in a truncated Nef protein of 20 amino acids. BK28*nef*56 contains a G to T transversion at position 9227 to give a stop codon (TGA) resulting in a truncated (56 amino acids) Nef protein. BK28Δ*nef*1 and

TABLE 1

Mutations Introduced into the SIVmac251-BK28 *nef* Gene

Mutant	Deletion	Nucleotide exchange	Frame shift	aa before frame shift	aa after frame shift	Length of Nef
BK28-wt	—	—	—	—	—	247 aa
BK28-*nef*20	—	9120 (T > A)	—	—	—	20 aa
BK28-*nef*56	—	9227 (G > T)	—	—	—	56 aa
BK28-Δ*nef*1	9226–9427	—	0	56	124	180 aa
BK28-Δ*nef*13	9226–9368	—	+1	55 (+1)[a]	33	55 aa

[a] Additional amino acid introduced due to the position of the deletion.

Δ*nef*13 carry deletions in the *nef* gene that neither overlap with *env* nor affect the polypurine tract immediately adjacent to the U3 region of the 5′ long terminal repeat. They were initially constructed to be tested as live vaccines in rhesus macaques. The nucleotide sequence of all mutated *nef* genes was confirmed by DNA sequencing. The molecular weights of the Nef proteins were checked by SDS–PAGE of immunoprecipitated, radioactively labeled proteins expressed in infected HuT78 cells. While the 247-amino acid (aa) Nef protein of the wild type could be detected, this 36-kDa protein was not detected in cells infected with the mutants *nef*20, *nef*56, and Δ*nef*13 (data not shown). The 180-aa Nef protein of mutant Δ*nef*1 could be detected (data not shown).

SIVmac251 viruses with defective *nef* genes show increased susceptibility to the noncytotoxic antiviral activity of CD8$^+$ lymphocytes

To study the effect of CD8$^+$ lymphocytes on the *in vitro* replication potential of wild-type and *nef*-defective viruses, unfractionated and CD8$^+$ T-cell-depleted PBMC of a healthy blood donor were infected with SIVmac251-wt and *nef* mutant viruses BK28*nef*56, BK28Δ*nef*1, and BK28Δ*nef*13 and cultured in the absence of IL-2 and exogenous stimulation (Fig. 2). The replication kinetics were followed for 28 days, and twice a week cell-free cell culture supernatant was removed for live virus determination. Full-length wild-type SIVmac251 replicated with similar kinetics in both cell populations. However, all *nef*-defective viruses, with the exception of the substitution mutant SIVmac251-*nef*56, replicated significantly better in CD8$^+$ T-cell-depleted human PBMC than in unfractionated human PBMC. The defects in the *nef* gene products therefore appear to render the virus susceptible to the negative influence of human CD8$^+$ T cells under these culture conditions. In the absence of IL-2, SIVmac251-*nef*56 replicated similarly in complete and CD8$^+$ T-cell-depleted human PBMC, albeit with reduced efficiency compared to those mutants with deletions in the *nef* gene.

IL-2 stimulates the noncytotoxic anti-HIV/SIV response of CD8$^+$ T cells

Simultaneously, the replication kinetics of the BK28-wt virus, carrying the complete *nef* open reading frame, and mutant viruses BK28*nef*56, BK28Δ*nef*1, and BK28Δ*nef*13 were compared on both unfractionated and CD8$^+$ T-cell-depleted human PBMC in the presence of IL-2 (Fig. 3). Under these conditions the replication kinetics of all viruses in CD8$^+$ T-cell-depleted PBMC were accelerated, with virus loads peaking at day 12 postinfection. In the absence of IL-2, virus

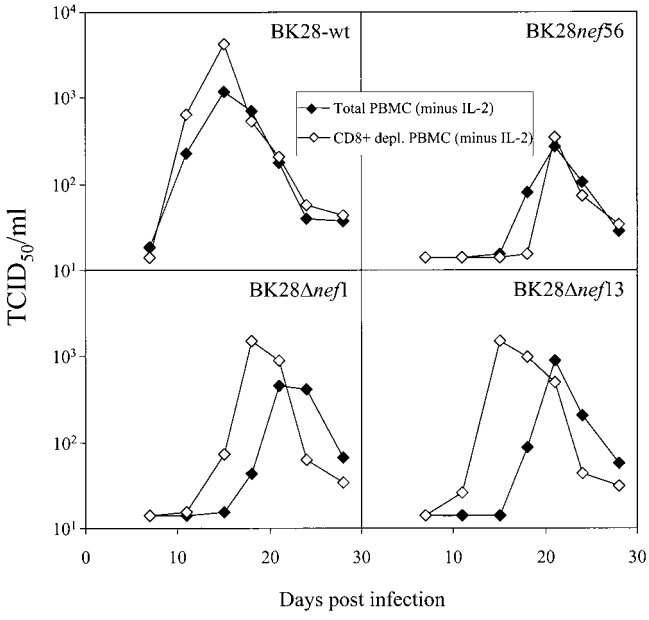

FIG. 2. Replication kinetics of SIVmac251-BK28-wt and *nef* mutant viruses in unstimulated, unfractionated versus CD8$^+$ T-cell-depleted human PBMC in the absence of IL-2. Unfractionated (2×10^7) and CD8$^+$ T-cell-depleted (1.8×10^7) human PBMC (both unstimulated) were infected with 2000 $TCID_{50}$ of the viruses BK28-wt, BK28*nef*56, BK28Δ*nef*1, and BK28Δ*nef*13. After washing, the cells were resuspended in 10 ml medium and cultured for 28 days in the absence of IL-2. Starting at 7 days postinfection, aliquots of the supernatant were removed twice weekly for determination of live virus titer. The results from one representative experiment are shown. The experiments were repeated three times using PBMC from different donors, with similar results.

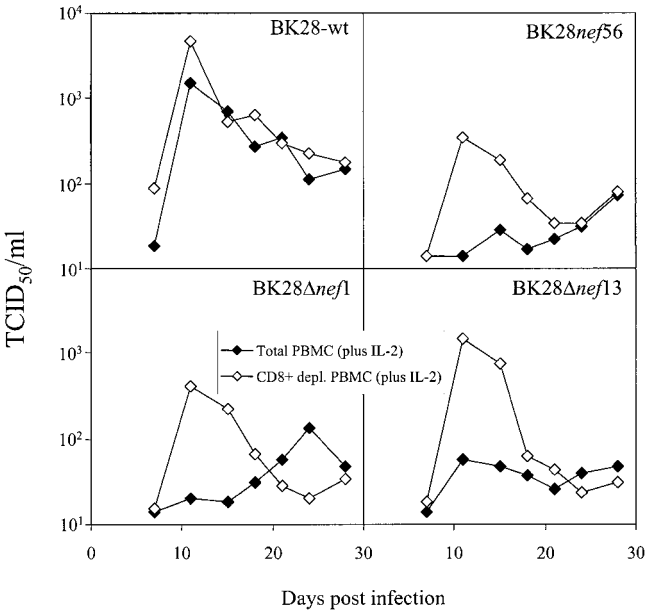

FIG. 3. Replication kinetics of SIVmac251-BK28-wt and *nef* mutant viruses in experimentally unstimulated human total and $CD8^+$ cell-depleted PBMC in the presence of IL-2. Total (2×10^7) and $CD8^+$ cell-depleted (1.8×10^7) human PBMC (both experimentally unstimulated) were infected with 2000 $TCID_{50}$ of the viruses BK28-wt, BK28*nef*56, BK28Δ*nef*1, and BK28Δ*nef*13. After washing, the cells were resuspended in 10 ml PBMC medium and cultured for 28 days in the presence of 80 U/ml IL-2. Starting at 7 days postinfection, aliquots of the supernatant were removed twice weekly for determination of live virus titer. The results from one representative experiment are shown. The experiments were repeated three times using PBMC from different donors, with similar results.

loads reached similar levels, but with slower kinetics (Fig. 2). However, whereas the wild-type virus replicated in unfractionated human PBMC to similar levels regardless of the presence or absence of IL-2 (Figs. 2 and 3), all *nef*-defective BK28 virus mutants replicated very poorly in the presence of IL-2. In particular, the replication of substitution mutant SIVmac251-*nef*56, similar in whole and $CD8^+$ T-cell-depleted PBMC cultures without IL-2 (Fig. 2), was severely inhibited in cultures of total PBMC in the presence of IL-2 (Fig. 3). The negative effect of $CD8^+$ T cells on the replication of *nef*-defective viruses in unfractionated human PBMC without exogenous IL-2 (Fig. 2) was even more pronounced in the presence of IL-2 (Fig. 3). It would therefore appear that IL-2 stimulates the anti-HIV/SIV activity of the $CD8^+$ T cells in such cultures. It should be noted that the presence of IL-2 in the culture medium of unstimulated human PBMC, either total or $CD8^+$ T cell depleted, resulted in an increase in the number of viable cells by a factor two or more (Fig. 4). It should be noted that although the increased susceptibility to the noncytotoxic antiviral activity of $CD8^+$ lymphocytes was observed on three different occasions using PBMC from random, unrelated blood donors, the effect occasionally was not observed. There were cases in which we could not detect a significant difference between the replication potentials of *nef*-defective viruses on total and $CD8^+$ T-cell-depleted PBMC cultures, possibly due to differences in CD8 factor production by (or *in vivo* activation status of) the cells. In other cases, even the wild-type virus replicated better in $CD8^+$ T-cell-depleted PBMC cultures than in total PBMC cultures.

PHA stimulation of unfractionated human PBMC overrides the suppression of viral replication by $CD8^+$ T cells

Unfractionated and $CD8^+$ T-cell-depleted human PBMC from the same donor were stimulated with phytohemagglutinin (PHA) for 3 days in the presence of exogenous IL-2 and then infected with BK28-wt virus carrying the complete *nef* open reading frame and mutant viruses BK28*nef*56, BK28Δ*nef*1, and BK28Δ*nef*13. All viruses grew well on both unfractionated and $CD8^+$ T-cell-depleted human PBMC (Fig. 5). PHA stimulation of the unfractionated human PBMC therefore appears to override the suppression of the viral replication mediated by $CD8^+$ T cells.

Target cells for viral replication in experimentally nonstimulated human PBMC cultures

SIVmac251-BK28-wt virus replicates in experimentally unstimulated primary T cells (total or $CD8^+$ T cell de-

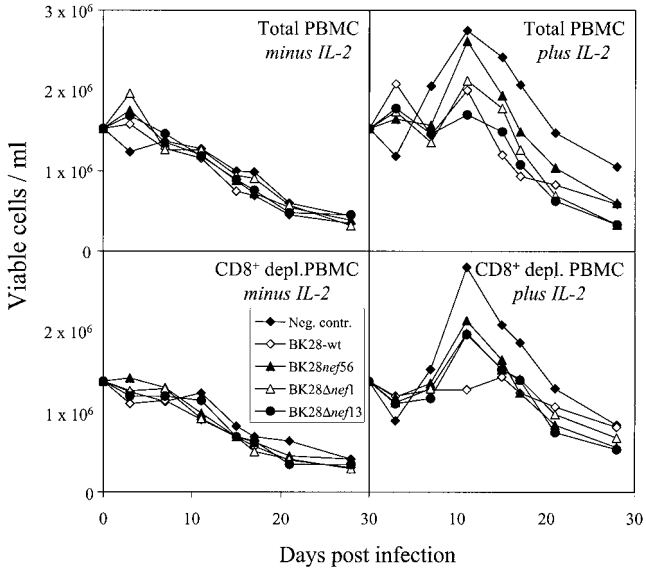

FIG. 4. Effects of IL-2 on viable cell numbers. Unfractionated and $CD8^+$ T-cell-depleted human PBMC (both unstimulated) were cultured in the presence and absence of IL-2 and infected with SIVmac251-BK28-wt and *nef* mutant viruses BK28*nef*56, BK28Δ*nef*1, and BK28Δ*nef*13. Cell viabilities were determined at different times using trypan blue staining. The results from one representative experiment are shown. The experiments were repeated three times using PBMC from different donors, with similar results.

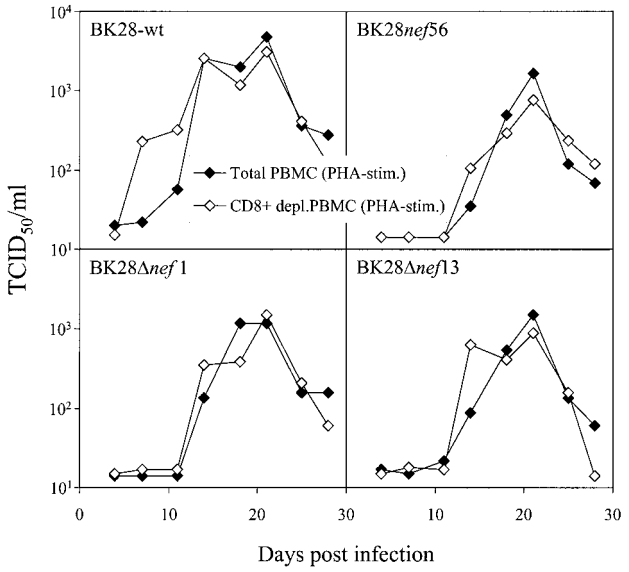

FIG. 5. Replication kinetics of SIVmac251-BK28-wt and *nef* mutant viruses in PHA stimulated human total versus CD8$^+$ cell-depleted PBMC in the presence of IL-2. Total (2×10^7) and CD8$^+$ cell-depleted (1.8×10^7) human PBMC (both stimulated for 3 days with PHA) were infected with 2000 $TCID_{50}$ of the viruses BK28-wt, BK28*nef*56, BK28Δ*nef*1, and BK28Δ*nef*13. After washing, the cells were resuspended in 10 ml PBMC medium and cultured for 28 days in the presence of 80 U/ml IL-2. Starting at 4 days postinfection, aliquots of the supernatant were removed twice weekly for determination of live virus titer. The results from one representative experiment are shown. The experiments were repeated three times using PBMC from different donors, with similar results.

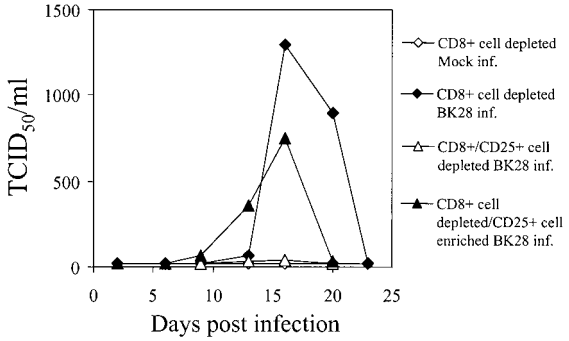

FIG. 6. Replication kinetics of SIVmac251-BK28-wt in CD8$^+$ T-cell-depleted, in CD8$^+$ CD25$^+$ T-cell-depleted, and in CD8$^+$ T-cell-depleted/CD25$^+$ T-cell-enriched non-mitogen-stimulated human PBMC. Approximately 1×10^7 each of CD8$^+$ T-cell-depleted, CD8$^+$ CD25$^+$ T-cell-depleted, and CD8$^+$ T-cell-depleted/CD25$^+$ T-cell-enriched non-mitogen-stimulated human PBMC were infected with 2000 $TCID_{50}$ SIVmac251-BK28-wt, resuspended in 5 ml PBMC medium, and cultured for 23 days in the absence of IL-2. Starting at 2 days postinfection, aliquots of the supernatant were removed twice weekly for determination of live virus titer.

pleted; Figs. 2 and 3). Does the BK28-wt Nef protein act as an activator of lymphocytes and hence of viral replication? While most lymphocytes in the blood are resting or minimally activated, HIV and SIV both require activated CD4$^+$ T cells for replication (McDougal *et al.,* 1985), the one clear exception being the acutely lethal pbj14 strain of SIV (Fultz, 1991). Activated CD4$^+$ and CD8$^+$ T lymphocytes express high-affinity IL-2 receptors (Waldmann, 1993), which can be detected using antibodies specific for the α-chain (CD25). By magnetic-activated cell sorting, three cell populations were prepared: (i) CD8$^+$ T-cell-depleted PBMC, (ii) CD8$^+$/CD25$^+$ T-cell-depleted PBMC, and (iii) CD8$^+$ T-cell-depleted/CD25$^+$ T-cell-enriched PBMC. These cells were infected with wild-type SIVmac251-BK28 virus without prior stimulation. BK28-wt virus replicated well both in CD8$^+$ T-cell-depleted human PBMC and in CD8$^+$ T-cell-depleted/CD25$^+$ T-cell-enriched human PBMC, but very poorly in PBMC depleted of both CD8$^+$ and CD25$^+$ T cells (Fig. 6). This confirms that prestimulated CD4$^+$ lymphocytes (CD4$^+$ CD25$^+$) harbor the target cells for virus propagation. Note that in the absence of exogenous IL-2 the viability of both the uninfected and infected CD8$^+$ CD25$^+$ cell-depleted human PBMC populations dropped rapidly compared to that in the other cultures (Fig. 7, panels C and D), apparently because this culture is deprived of almost all of its activated (CD25$^+$) cells that have the potential to expand in the presence of IL-2. This culture has two handicaps: missing CD25$^+$ cells and the absence of IL-2.

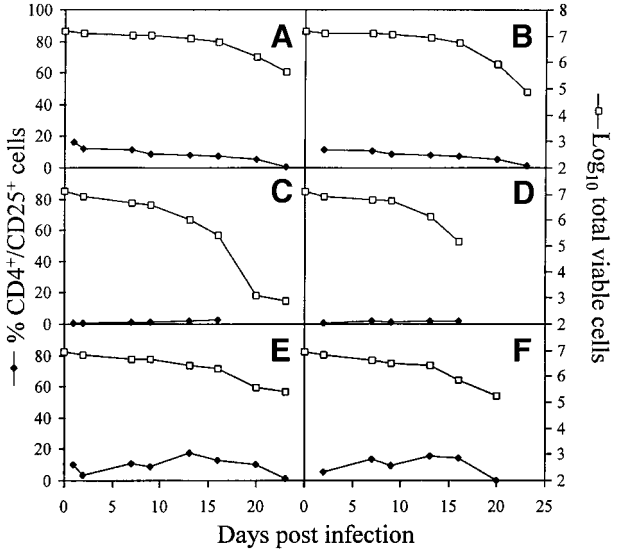

FIG. 7. Viable cell numbers and percentages of activated (CD25$^+$) CD4$^+$ T cells in cultures of CD8$^+$ T-cell-depleted, CD8$^+$ CD25$^+$ T-cell-depleted, and CD8$^+$ T-cell-depleted/CD25$^+$ T-cell-enriched unstimulated human PBMC infected with SIVmac251-BK28-wt. CD8$^+$ T-cell-depleted (A, B), CD8$^+$ CD25$^+$ T-cell-depleted (C, D), and CD8$^+$ T-cell-depleted/CD25$^+$ T-cell-enriched (E, F) unstimulated human PBMC were mock infected (A, C, E) or infected with 2500 $TCID_{50}$ SIVmac251-BK28 (B, D, F). After washing, cells were resuspended in 5 ml medium and cultured for 23 days in the absence of IL-2. Starting at 2 days postinfection, aliquots of the supernatant were removed twice weekly. Viable cell numbers were determined by trypan blue staining, and cells were prepared for cell surface CD4$^+$ CD25$^+$ FACS analysis as described under Materials and Methods.

Increase of activated CD4$^+$ T cells in human PBMC cultures

FACS analysis of freshly isolated PBMC revealed comparable amounts of CD4$^+$ CD25$^+$ in the total (3%; absolute number, 6 $\times$ 10^5) and in the CD8$^+$ T-cell-depleted cultures (3.9%; absolute number, 7 $\times$ 10^5) in the gated lymphocyte populations. Using FACS analysis we determined an increase of activated (CD25$^+$) CD4 T cells in unfractionated and CD8$^+$ T-cell-depleted PBMC cultures in the presence and absence of exogenous IL-2 throughout the course of the experiment (Fig. 8), demonstrating comparable amounts of potential target cells in the corresponding total and fractionated PBMC cell culture systems.

Increase of activated CD8$^+$ T cells in human PBMC cultures

Using FACS analyses we determined the increase of activated CD8$^+$ T cells in unfractionated and CD8$^+$ T-cell-depleted human PBMC cultures in the presence and absence of exogenous IL-2 throughout the course of the experiment (Fig. 9). CD8$^+$ T cell depletion resulted in a 20-fold decrease in the number of cells expressing high levels of this surface molecule. In unfractionated PBMC cultures the presence of IL-2 led to a significant increase in activated CD8$^+$ T cells (Fig. 9).

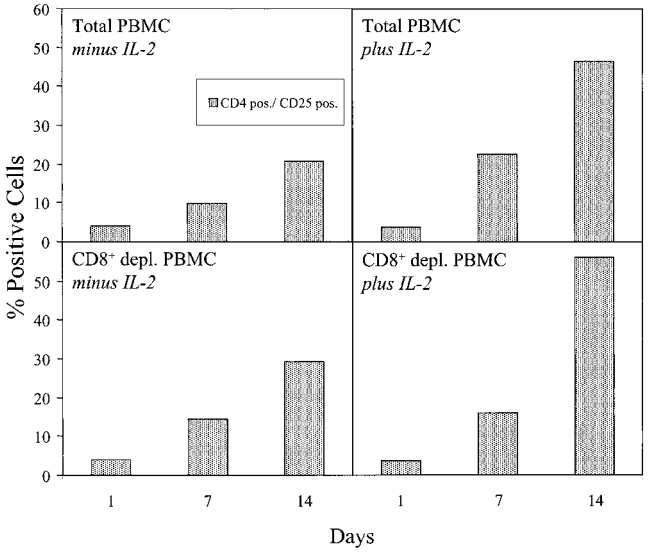

FIG. 8. Kinetics of activated (CD25$^+$) CD4$^+$ T cells in human PBMC cultures. Total and CD8$^+$ T-cell-depleted human PBMC were cultured for 28 days in the presence and absence of IL-2. At 1, 7, and 14 days postisolation, aliquots of the cell cultures were removed and cells were prepared for cell surface CD4$^+$ CD25$^+$ FACS analysis as described under Materials and Methods.

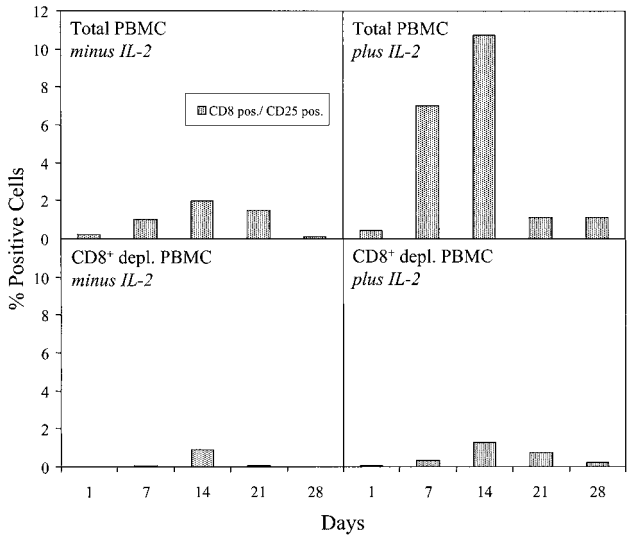

FIG. 9. Kinetics of activated (CD25$^+$) CD8$^+$ T cells in human PBMC cultures. Total and CD8$^+$ T-cell-depleted human PBMC were cultured for 28 days in the presence and absence of IL-2. At 1, 7, 14, 21, and 28 days postisolation, aliquots of the cell cultures were removed and cells were prepared for cell surface CD8$^+$ CD25$^+$ FACS analysis as described under Materials and Methods.

Effect of the β-chemokines MIP-1α, MIP-1β, and RANTES and of IL-16 on the replication of SIVmac251-BK28-wt virus and *nef* mutant BK28*nef*20 in total human PBMC

We next determined the effect of known antiviral effectors derived from CD8$^+$ T cells on the replication potential of BK28-wt virus and *nef* mutant BK28*nef*20 (Fig. 10). Total PBMC were infected with BK28-wt and *nef* mutant BK28*nef*20 in the presence of a cocktail of neutralizing antibodies against the β-chemokines MIP-1α, MIP-1β, and RANTES (Cocchi *et al.,* 1995, 1996) and IL-16, respectively. The presence of neutralizing antibodies against IL-16 had no significant effect on the replication kinetics of either the BK28-wt virus or the BK28*nef*20 virus (Fig. 10). The presence of neutralizing antibodies against the β-chemokines MIP-1α, MIP-1β, and RANTES increased the replicative capacity of both the BK28-wt virus and the *nef* mutant BK28*nef*20 tremendously (Fig. 10), suggesting that the inhibition of viral replication by β-chemokines and the inhibitory effect of CD8$^+$ lymphocytes on *nef* mutant SIVmac viruses are harbored on different levels.

DISCUSSION

Nef and the activation state of the target cells permissive for viral replication

We studied the effect of the CD8$^+$ cell-derived HIV/SIV-suppressing activity on wild-type and *nef* mutant viruses in an *in vitro* replication system. SIVmacBK28-wt virus could replicate well in PBMC cultures which had not

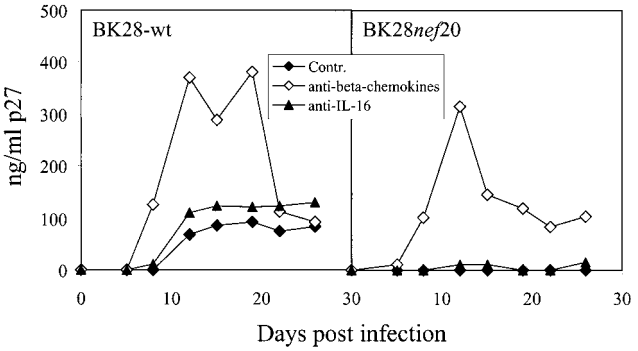

FIG. 10. Replication kinetics of SIVmac251-BK28-wt and *nef* mutant BK28*nef*20 in the presence of neutralizing antibodies against the β-chemokines MIP-1α, MIP-1β, and RANTES and IL-16. Total human PBMC (2×10^6) were incubated in the presence of a cocktail of neutralizing antibodies against β-chemokines MIP-1α, MIP-1β, and RANTES for 2 h. The same amount of total human PBMC was incubated in the presence of a neutralizing antibody against IL-16. After a preincubation time of 2 h, cells were infected with 500 $TCID_{50}$ of SIVmac251-BK28-wt and *nef* mutant virus BK28*nef*20. After washing, the cells were resuspended at a cell density of 2×10^6 cells/ml in PBMC medium and cultured for 28 days in the presence of neutralizing antibodies against the β-chemokines MIP-1α, MIP-1β, and RANTES and IL-16 and in the presence of 80 U/ml IL-2. Starting at 4 days postinfection, aliquots of the supernatant were removed twice weekly for determination of the amount of viral p27 gag protein. The results from one representative experiment are shown. The experiments were repeated twice using PBMC from different donors, with similar results.

been experimentally stimulated with mitogen. While coreceptor expression (CCR5 in the case of SIVmac251; Chakerian *et al.,* 1997) is required for viral entry into CD4$^+$ cells, productive infection requires cellular activation and entry into the G_1b phase of the cell cycle (Zack *et al.,* 1990; Korin and Zack, 1998). Therefore, replication of HIV/SIV occurred, at least initially, in cells naturally activated *in vivo* before removal from the body and thus resembling the physiologic situation *in vivo.* Approximately the same amounts of activated (CD25$^+$) CD4$^+$ cells were detected in the unfractionated and CD8$^+$ T-cell-depleted cell cultures, indicating that comparable numbers of target cells permissive for SIV replication were present in both culture systems.

It has been postulated that the major function of Nef is to stimulate resting cells after infection to allow virus propagation. This idea has gained further support by the observation that the ability of the acutely pathogenic SIVpbj14 to activate and grow in unstimulated PBMC cultures is due to the presence of a second YXXL motif in Nef with the characteristics of an SH2 binding domain (Fultz, 1991; Du *et al.,* 1995). Indeed, site-directed mutagenesis of SIVmac239 *nef* to yield a second YXXL motif was sufficient to impart the pbj14 phenotype both *in vitro* and *in vivo* (Du *et al.,* 1995). We have shown here that SIVmac-BK28 is able to replicate well in PBMC in the absence of exogenous mitogen or IL-2 and that defects in Nef reduce this capability. On the face of it, this would seem to support a stimulatory role for Nef. However, removal of CD25$^+$ cells from cultures of CD8$^+$ T-cell-depleted PBMC virtually prevented replication of the wild-type virus in the absence of exogenous IL-2 (Fig. 6). Furthermore, cytokine signals seem to be sufficient for HIV infection of resting T lymphocytes, since resting cells stimulated with the cytokine IL-2, IL-4, IL-7, or IL-15 could be infected with a replication-competent CCR5 tropic HIV-1 (Unutmaz *et al.,* 1999), albeit with a low efficiency.

The presence of an intact BK28 Nef is insufficient to permit replication in truly resting T cells. In this context, it is important to note that the suppressive effect of CD8$^+$ T cells was not observed with PHA-stimulated unfractionated human PBMC (Fig. 5). The experimental finding that PHA stimulation of unfractionated human PBMC overrides the suppression of viral replication by CD8$^+$ T cells seems to be in contrast to the fact that PHA is known to activate this antiviral activity. Doubtlessly, PHA stimulation provides a large reservoir of activated target cells. But, since there is a role of Nef in modulating T cell activation events in the target cell, one could speculate that stimulation by PHA renders the stimulated cell with no nef function needed (wt and nef mutant viruses do not behave differently). Indeed, experimental evidence suggests that Nef can promote T cell activation (Baur *et al.,* 1994; Du *et al.,* 1995; Alexander *et al.,* 1997; Hanna *et al.,* 1998). Recently, it has been reported that HIV-1 Nef increases T cell activation in a stimulus-dependent manner (Schrager and Marsh, 1999). It has to be noted that activation of T cells, as defined by IL-2 generation, stimulated with classical chemical mitogens was unaffected by the expression of Nef (Schrager and Marsh, 1999). However, Nef increased IL-2 secretion when cells were stimulated through the T cell receptor and the CD28 coreceptor (Schrager and Marsh, 1999). Studies of HIV infection of primary cells typically require the use of mitogens such as PHA. Schrager and Marsh (1999) deduced that under these conditions the enhancing activity of Nef may not be evident. In addition, they showed that Nef lowers the threshold of the dual-receptor T cell activation pathways because the increase in activation is caused by an increase in the number of cells reaching full activation. Furthermore, Wang *et al.,* (2000) published that the Nef-mediated induction of IL-2 reflects the activation of the transcription factors NF-AT and NFκB. Very recently, it was reported that infection with HIV leads to selective transcription of the *tat* and *nef* genes before integration in quiescent cells, leading to increased T cell activation (Wu and Marsh, 2001). Artificial stimulation of PBMC by PHA should be avoided in assays designed to identify putative antiviral factors.

Control of viral replication *in vitro*

The data presented here suggest that one positive effect on viral replication mediated by an intact *nef* is the abrogation of the noncytotoxic anti-HIV/SIV activity of

$CD8^+$ T cells. The replication of SIVmac251-BK28 mutants expressing defective *nef* gene products was found to be suppressed by human $CD8^+$ T cells, whereas the full-length virus was not. Under the conditions used, the Nef protein of SIVmac251-BK28 seemed to counteract the anti-HIV/SIV activity of $CD8^+$ T cells. This effect was accentuated in unstimulated, unfractionated human PBMC by the addition of exogenous IL-2, which therefore appears to stimulate the antiviral effect of $CD8^+$ T cells. This is in agreement with data showing IL-2 induction of $CD8^+$ T-cell-mediated suppression of HIV replication in $CD4^+$ T cells and a corresponding abrogation of the stimulatory effect of this cytokine on virus expression (Kinter *et al.,* 1995). The presence of exogenous IL-2 induced a quantitative increase in activated $CD8^+$ cells, particularly in the unfractionated PBMC cultures (Fig. 9). Besides this, IL-2 appears to induce a qualitative increase in the HIV-1-suppressing activity of activated ($CD25^+$) $CD8^+$ cell populations (Kinter *et al.,* 1995).

Effect of chemokines

Primate lentiviruses use chemokine receptors in addition to the CD4 receptor to initiate virus infection. SIV uses CCR5 as coreceptor (Chackerian *et al.,* 1997), and the native ligands for CCR5, the β-chemokines MIP-1α, MIP-1β, and RANTES, specifically inhibit infection of SIV. The mechanism of HIV/SIV inhibition by chemokines is complex. In addition to blocking viral entry by competition for receptor occupancy, downregulation of coreceptors as a result of chemokine binding reduces the efficiency of viral entry (Trkola *et al.,* 1998). Furthermore, signals transduced into the cell by the receptor interactions of chemokines might interfere with transcription of the viral genome (Littman, 1998).

The presence of neutralizing antibodies against the β-chemokines in our replication kinetics (infection of $CD8^+$ T-cell-depleted PBMC with SIVmac-BK28 and SIVmac-*nef*20) had tremendous effects on the replication potential of both wild-type and *nef* mutant virus. If the β-chemokines MIP-1α, MIP-1β, and RANTES would merely be responsible for the increased susceptibility of the *nef*-defective SIVmac virus mutants to the noncytotoxic antiviral activity of $CD8^+$ lymphocytes, the presence of neutralizing antibodies against these β-chemokines would increase the replication potential of the *nef* mutants up to the levels detected in $CD8^+$-depleted PBMC cultures. But what we see is a tremendous augmentation of the replicative capacity of both the BK28-wt virus and the *nef* mutant BK28*nef*20 in the presence of neutralizing antibodies against the β-chemokines. We conclude that these two effects (inhibition by β-chemokines and the inhibitory effect of $CD8^+$ lymphocytes on *nef* mutant SIVmac viruses) are harbored on different levels. Furthermore, it has been reported that expression of the coreceptor CCR5 on T cells is upregulated by exposure to IL-2 *in vitro* (Trkola *et al.,* 1996; Bleul *et al.,* 1997) and *in vivo* (Zou *et al.,* 1999).

Control of viral replication *in vivo*

The observation that rhesus macaques infected with SIVmac carrying large deletions in the *nef* gene show lower virus loads than wild-type infected animals (Kestler *et al.,* 1991) and the finding that depletion of $CD8^+$ cells in such animals leads to a dramatic increase in plasma viremia (Metzner *et al.,* 2000) suggest that $CD8^+$ cells play an important role in controlling viral replication. Inhibition of SIV replication by $CD8^+$ T cells from animals immunized with live attenuated SIV strains involves both MHC-restricted and MHC-unrestricted mechanisms, and the latter is due principally to soluble factors other than RANTES, MIP-1α, and MIP-1β (Gauduin *et al.,* 1998). There is evidence that cytotoxic T lymphocytes (CTLs) are critical in controlling virus replication. Low viral loads in HIV-1-infected long-term nonprogressors (Cao *et al.,* 1995; Dyer *et al.,* 1999) and in rhesus macaques infected with SIVΔ*nef* viruses (Johnson *et al.,* 1997) have been associated with high levels of virus-specific CTL activity. Recently, evidence for the *in vivo* control of wild-type SIV replication during acute and chronic infection has been obtained (Jin *et al.,* 1999; Schmitz *et al.,* 1999).

In vivo, Nef may confer a growth advantage to the virus by downregulating class I MHC (Schwartz *et al.,* 1996) and thereby enabling infected cells to avoid CTL killing (Collins *et al.,* 1998). As a consequence of this, *nef*-defective viruses would be more susceptible to lysis by CTL. Refering to our own data, *nef*-defective viruses are more susceptible to the antiviral activity of $CD8^+$ cells *in vitro,* and if this is also the case *in vivo* it could account for the suppressed virus loads seen in SIVmacΔ*nef*-infected animals.

Why do SIVmac251-BK28 *nef* mutant viruses show increased susceptibility to the noncytotoxic antiviral activity of $CD8^+$ lymphocytes?

The molecular mechanism underlying our observation that defects in the *nef* gene render primate lentiviruses more susceptible to the noncytotoxic antiviral activity of $CD8^+$ lymphocytes remains to be elucidated. To answer the question of whether a soluble factor is involved, we performed experiments with a double-chamber system, providing $CD8^+$ T cells separated by a membrane from the $CD8^+$ T-cell-depleted PBMC culture (data not shown). We did not see a significant difference between the replication of the *nef*-defective viruses in the $CD8^+$ T-cell-depleted PBMC culture and that in the double-chamber system. Replication in unfractionated PBMC was hampered. Our interpretation of this result is that the functioning of a soluble factor(s) cannot be ruled out. Maybe close cell–cell contact is necessary to allow function of a soluble factor(s).

The increased susceptibility of *nef* mutants to the antiviral effects of CD8$^+$ T cells, while being a new observation, could be the result of the previously described Nef functions. One possible explanation (although there is no direct evidence for this hypothesis) could involve downregulation of the CD4 molecule at the cell surface by Nef (Garcia and Miller, 1991; Aiken *et al.,* 1994; Rhee and Marsh, 1994; Sanfridson *et al.,* 1994). All of the BK28 Nef mutant proteins used in this study lack this ability (data not shown). Removal of their cell surface receptors is a common feature of retroviruses and is usually mediated by the viral Env proteins. In contrast, Nef induces the internalization and degradation of preexisting cell surface CD4 and therefore has the potential to act far more rapidly, particularly as Nef is an early protein. As well as hindering superinfection by other viruses (Benson *et al.,* 1993) and facilitating the release of virus particles, removal of CD4 would block any antiviral signal entering the cell via this molecule. CD4 is physically associated with the protein tyrosine kinase p56lck, which supports the notion that this integral membrane glycoprotein participates in signal transduction (Rudd *et al.,* 1988; Veillette *et al.,* 1989; Weiss and Littman, 1994). Furthermore, cross-linking of cell surface CD4 by certain monoclonal antibodies has been shown to inhibit HIV replication in the cell at the level of expression (Benkirane *et al.,* 1993). As defects in Nef render the virus more susceptible to the antiviral effect of CD8$^+$ cells, it is possible that one effect of an intact Nef is to abrogate one or more of the CD8$^+$ T cell suppressor factors by the removal of CD4. IL-16, a candidate antiviral factor secreted by CD8$^+$ T cells (Baier *et al.,* 1995, 1997), is clearly not responsible for the observed effect.

In conclusion, the SIVmac Nef protein was shown to have the additional function of abrogating the suppressive effect of antiviral factors produced by human CD8$^+$ cells. Although derived from an *in vitro* test system, this phenomenon, if occurring *in vivo,* could further explain the attenuated phenotype of Nef-deletion mutants in macaques. Ongoing experiments using a panel of HIV-1 deletion mutants should allow more light to be shed on this and the many other functions of *nef.*

MATERIALS AND METHODS

Construction of SIVmac *nef* mutants

The biologically active proviral SIVmac251 DNA clone BK28 (Kornfeld *et al.,* 1987), was kindly provided by J. Mullins (University of Washington, Seattle, WA) (GenBank Accession No. X06393). Plasmid pPCR*nef*-wt, starting at nucleotide 8724 and comprising the complete 3′ end of the proviral genome, was generated by PCR with BK28 as a template using the primers A8724(+) 5′-CGATGAATTCTCTAGA**GCTAGCTAAGTTAAGGCAGGGG**-3′ corresponding to positions 8724 to 8745 of SIVmac251-BK28 (in bold) and B10250(−) 5′-CGATGAATTC**ATGCTAGGGATTTTCCTGCTTCGG**-3′ corresponding to positions 10250 to 10227 of SIVmac251-BK28 (in bold). Both contain an *Eco*RI restriction endonuclease site (underlined). The plasmid pPCR*nef*-wt served as a template for the following constructs.

Construction of *nef* substitution mutants

Substitution *nef* mutant BK28*nef*56 was generated by PCR assembly using two PCR amplified ds fragments with homologous regions. The homologous region contained a single nucleotide exchange from G to T at position 9227. Fragment A was amplified using the primer pair A8724(+) and A9232(−) 5′-CTGTCACTCACAAGAGCGTGAGCTCAAG-3′, the latter corresponding to positions 9232–9204 of SIVmac251-BK28 (the nucleotide exchange at position 9227 is underlined). Fragment B was amplified using primer pair B9211(+) 5′-CTCACGCTCTTGTGAGTGACAGAAATAC-3′, corresponding to positions 9211–9239 of SIVmac251-BK28 (the nucleotide exchange at position 9227 is underlined) and B10250(−). The PCR fusion product of fragments A and B using the outer primer pairs A8724(+) and B10250(−) was purified, digested with *Eco*RI, and inserted into the *Eco*RI site of vector pUC18 (pPCR*nef*56). The substitution *nef* mutant BK28*nef*20 was generated by PCR assembly. The homologous region contained a single nucleotide exchange from T to A at position 9120. Fragment A was amplified using the primer pair A8724(+) and A9125(−) 5′-CCCGCTAGAGTTTCTGTCGCAGAT-3′, the latter corresponding to positions 9125 to 9101 of SIVmac251-BK28 (the nucleotide exchange at position 9120 is underlined). Fragment B was amplified using primer pair B9106(+) 5′-GCGACAGAAACTCTAGCGGGCGCGT-3′, corresponding to positions 9106–9130 of SIVmac251-BK28 (the nucleotide exchange at position 9120 is underlined) and B10250(−). The PCR fusion product of fragments A and B using the outer primer pairs A8724(+) and B10250(−) was purified, digested with *Eco*RI, and inserted into the *Eco*RI site of vector pUC18 (pPCR*nef*20).

Construction of Nef deletion mutants

Nef deletion mutant BK28Δ*nef*1 was also created by PCR assembly. Briefly, fragment A was amplified using primer A8724(+) and the assembly primer A9446(−) 5′-**CCAGTCCCCCCTTTTCTTTT**TCACAAGAGCGTGAGCTCAAG-3′ (SIVmac251-BK28 sequence 9446–9427 is in bold and 9225–9205 is underlined). Fragment B was amplified using the assembly primer B9222(+) 5′-**GTGA**AAAAGAAAAGGGGGGACTGGAAGGG-3′ (SIVmac251-BK28 sequence 9222–9225 is in bold and 9427–9451 is underlined) together with primer B10250(−). PCR fusion of fragments A and B using the outer primer pair A8724(+) and B10249(−), restriction with *Eco*RI, and cloning into the *Eco*RI site of pUC18 resulted in

pPCRΔ*nef*1, which contains a *nef* gene missing precisely the sequences between position 9225 and 9427. Construction of the deletion mutant BK28Δ*nef*13 followed the same protocol except that the assembly primer A9387(−) 5′-**GCTCTTAGGGGAACTTTTG**TCACAAGAGCGTGAGCTCAAG-3′ (SIVmac251-BK28 sequence 9387–9369 is in bold and 9225–9205 is underlined) was used as the 3′ primer for fragment A and primer B9369(+) 5′-**CAAAAGTTCCCCTAAGAGCAATG**-3′ (SIVmac251-BK28 sequence 9369–9391 is in bold) as the 5′ primer for fragment B. The resulting plasmid pPCRΔ*nef*13 had the *nef* gene sequences from positions 9225 to 9368 deleted.

The molecular weights of the Nef proteins were checked by SDS–PAGE of immunoprecipitated, radioactively labeled proteins (data not shown).

Transfection and production of virus stocks

SW480 cells [American Type Culture Collection (ATCC), Rockville, MD] were grown at 37°C in Dulbecco's modification of Eagle's medium supplemented with 10% (v/v) fetal calf serum (FCS). The different *Nhe*I-*Eco*RI fragments of the mutated *nef* genes of BK28 were ligated to the 15-kb pBK28 *Nhe*I-*Eco*RI vector fragment and used to transfect subconfluent cultures of SW480 cells using the LipofectAMINE reagent (Life Technologies, Gaithersburg, MD). Two days posttransfection, cultures were cocultivated with HuT78 cells for 48 h. Infected HuT78 cells were subsequently removed and maintained in RPMI 1640 supplemented with 10% (v/v) fetal calf serum. Cell-free culture supernatant from permanently infected HuT78 cultures was passed through a 0.22-μm-pore-size filter and stored as aliquots in liquid nitrogen. Titration of the virus stocks was performed by endpoint dilution on C8166 cells (ATCC).

Quantification of virus infectivity

Virus-containing supernatants were serially diluted in media and aliquoted (50 μl per well, eight replicates per dilution) into U-well microtiter plates containing 2000 C8166 cells in 150 μl. After 7 days incubation at 37°C, cells and viruses were lysed by the addition of 20 μl 2% Tween 20, and 50 μl from each well was transferred to the corresponding well of an ELISA plate for detection of the viral Gag protein using a p27 antigen capture assay. The number of positive wells was then used to determine the $TCID_{50}$ of the original sample using the Spearmann–Karber method.

Viral protein analysis

Permanently infected HuT78 cells were metabolically labeled with [^{35}S]methionine (30 μCi/ml) for 4 h and lysed in radioimmunoprecipitation assay (RIPA) buffer [150 mM NaCl, 5 mM Tris–Cl (pH 7.9), 10 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate]. Nef proteins were immunoprecipitated using a rabbit polyclonal Nef-specific antiserum raised against *Escherichia coli*-derived BK28 Nef and then subjected to SDS–PAGE.

Cell culture and infections

For each experiment, PBMC were isolated from the peripheral blood of a seronegative donor by Ficoll–diatricoate (Histopaque, Sigma, Deisenhofen, Germany) density gradient centrifugation and maintained in RPMI 1640 supplemented with 20% FCS, 1 mM glutamine with and without 80 U/ml recombinant IL-2. Half of the isolated PBMC were used as unfractionated culture and the other half were depleted of $CD8^+$ T cells using CD8 microbeads. Both cultures were resuspended in the same volume of medium, resulting in the same concentration of potential target cells. For stimulation, cells were cultivated for 1 day in the presence of 18 μg/ml PHA, washed in medium, and further cultivated in the presence of 80 U/ml IL-2. To study replication kinetics, 2×10^7 unfractionated and 1.8×10^7 $CD8^+$ T-cell-depleted PBMC (with and without PHA stimulation) were infected with 2000 $TCID_{50}$ of the different viruses for 4 to 8 h. After being washed three times in medium, the cells were cultured for 28 days in the presence or absence of IL-2. Twice weekly, aliquots of the supernatant were removed and the titer of infectious virus was assayed immediately as described above. Cells were refed with fresh medium, and viable cell numbers were determined by trypan blue staining.

Infections in the presence of neutralizing antibodies

Unfractionated and $CD8^+$ T-cell-depleted PBMC were prepared as described above and preincubated with neutralizing antibodies (NAb) against human MIP-1α, MIP-1β, and RANTES (R&D Systems, Minneapolis, MN) for 2 h before the start of the infection. The anti-RANTES NAb was used at 1.5 μg/ml (10-fold excess), the anti-MIP-1α NAb at 150 μg/ml (2-fold excess) and the anti-MIP-1β NAb at 10 μg/ml (10-fold excess). The anti-IL-16 NAb (PharMingen, San Diego, CA) was used at a final concentration of 10 μg/ml (10-fold excess). Levels of MIP-1α, MIP-1β, RANTES, and IL-16 in cell supernatants were measured with enzyme-linked immunosorbent assay kits from R&D Systems according to the manufacturer's instructions. Antibody concentrations were maintained for the duration of the experiment.

Purification of T lymphocyte subsets

Freshly isolated PBMC from a healthy blood donor were washed in medium and half were depleted of $CD8^+$ T cells using CD8 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) following the manufacturer's instructions. For $CD25^+$ cell depletion, $CD8^+$ T-cell-depleted PBMC were incubated at 4°C with an anti-CD25 monoclonal antibody (anti-IL-2R α-chain; Becton Dickin

son, San Jose, CA). After washing, cells were incubated with biotin-labeled goat antibody specific for mouse IgG $F(ab')_2$ (Dianova, Hamburg, Germany), washed again, and incubated with both super-paramagnetic microbeads conjugated with streptavidin (Miltenyi Biotech) and microbeads conjugated with goat anti-mouse IgG (Miltenyi Biotech) to allow possible cross-linking. Magnetic-activated cell sorting was then performed according to the manufacturer's instructions.

Cell surface marker analysis

Cells were stained simultaneously with phycoerythrin-conjugated anti-CD4 (Leu3a, Becton Dickinson) or anti-CD8 mAb (Leu2a, Becton Dickinson) and fluorescein isothiocyanate-conjugated anti-CD25 mAb (anti-IL-2Ra, Becton Dickinson) for 40 min on ice in the dark. Percentages of CD4/CD25- and CD8/CD25-positive T cells were determined by FACS analysis (FACScan; Becton Dickinson, Heidelberg, Germany) of gated lymphocyte populations.

ACKNOWLEDGMENTS

We thank Nicole Severing, Tanja Haase, and Julia Seidl for their expert technical assistance, Martin Selbert for sequencing, and Daniela Wesch, Christoph Conrad, and Andrea Glatzel for help with FACS analysis and many helpful discussions. This work was supported in part by contract No. 1506/TG04 to R.K. from the Federal Ministry of Health.

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